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Comparison of the In-House Made Carba-NP and Blue-Carba Tests: Considerations for Better Detection of Carbapenemase-producing *Enterobacteriaceae*

João Pires,[1,2] Regula Tinguely,[1] Bodmer Thomas,[3] Francesco Luzzaro,[4] and Andrea Endimiani [1*]

[1] Institute for Infectious Diseases, University of Bern, Bern, Switzerland; [2] Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland; [3]labormedizinisches zentrum Dr Risch, Department of Medical Microbiology, Bern-Liebefeld, Switzerland; [4]Laboratory of Microbiology, A. Manzoni Hospital, Lecco, Italy

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*__Corresponding authors__:
Prof. Andrea Endimiani MD, PhD
Institute for Infectious Diseases, University of Bern
Friedbühlstrasse 51, CH-3010, Bern, Switzerland
Phone: +41-31-632-8632; Fax: +41-31-632-8766
Emails: andrea.endimiani@ifik.unibe.ch; aendimiani@gmail.com

ABSTRACT

The in-house Carba-NP and Blue-Carba tests were compared using 30 carbapenemase- and 33 non-producing *Enterobacteriaceae*. Tests were read by three operators. 100% sensitivity was reported for both tests, but Carba-NP was slightly more specific than Blue-Carba (98.9% *vs.* 91.7%). We describe potential sources of error during tests' preparation and reading.

The continuous worldwide expansion of carbapenemase-producing *Enterobacteriaceae* (CPE) is a serious concern as infections caused by these pathogens have an increased mortality, morbidity, and associated health-care costs (Tängdén and Giske, 2015). Treatment options for CPE infections are often limited, since these organisms usually co-carry resistant determinants to other classes of antibiotics (Tängdén and Giske, 2015). Moreover, the heterogeneity of carbapenemase classes and types leads to a multiplicity of diverse carbapenem hydrolytic efficiencies and resistance phenotypes (Hrabák et al., 2014, Tängdén and Giske, 2015). Since carbapenem resistance mediated by carbapenemase production is continuously rising in *Enterobacteriaceae*, rapid, inexpensive, and reliable methods are urgently needed to identify CPE (Dortet et al., 2014).

Carba-NP and Blue-Carba are recent quick biochemical methods that detect carbapenemase activity when the enzyme breaks imipenem's β-lactam ring, leading to a pH decrease and consequent color shift of the pH-indicator in solution (Nordmann et al., 2012, Pires et al., 2013). Both methods proved to be fast (detection observed ≤2 hours), highly sensitive, specific and very cheap. Further studies have evaluated both tests, emphasizing their reproducibility, high sensitivity and specificity (Pasteran et al., 2015, Vasoo et al., 2013). However, others have questioned the utility of these methodologies (Tijet et al., 2013). Moreover, studies comparing the performance of the two tests are still scarce and those evaluating the impact of operators' experience in reading and interpreting results are lacking.

Since commercial tests have been just launched into the market (Novais et al., 2015, Poirel and Nordmann, 2015), we aim to compare the in-house made Carba-NP and Blue-Carba tests using a characterized collection of carbapenemase producing and non-producing *Enterobacteriaceae* in order to further identify potential sources of error.

Sixty-one previously characterized *Enterobacteriaceae* from different sources and countries (CPE, n=30, including 9 NDM, 10 OXA-48, 5 KPC, 3 NDM plus OXA-48, 2 VIM, and 1 IMP producers; non-CPE, n=33) recovered from cation adjusted Mueller-Hinton agar (Becton-Dickinson) were tested using Carba-NP and Blue-Carba, as previously described (Nordmann, Poirel and Dortet, 2012, Pires, Novais and Peixe, 2013). Both assays were executed in parallel two times each in non-consecutive days. Tests were performed and read by two different operators with previous experience in both assays (OP1 and OP2); a third operator (OP3) with no previous experience also read the results. Results were reported after 2 hours. Operators were blind regarding the species and *bla* gene content. Positive results were classified as "+", weak positive; "++", positive; and "+++", strong positive. MICs for imipenem, meropenem and ertapenem were assessed using Etest (bioMérieux) or microdilution ESB1F panels (Trek Diagnostics Systems).

As shown in Table 1, an overall sensitivity of 100% was obtained for both assays; however, Carba-NP revealed a higher specificity than Blue-Carba (98.9% *vs*. 91.7%, respectively). These high sensitivity and specificity for both tests are consistent with previous reports (Pasteran, Veliz, Ceriana, Lucero, Rapoport, Albornoz, Gomez and Corso, 2015, Pires, Novais and Peixe, 2013, Vasoo, Cunningham, Kohner, Simner, Mandrekar, Lolans, Hayden and Patel, 2013, Yusuf et al., 2014).

For Carba-NP, interpretation was more homogeneous, with OP1 interpreting correctly all isolates, while OP2 and OP3 identified one false-positive result only in the first assay. Blue-Carba's interpretation was similar for OP1 and OP2, whereas OP3 interpreted more false-positive results yielding a lower specificity (i.e., 96.9% for OP1 and OP2 *vs*. 89.4% for OP3). Nevertheless, false-positive results read by OP3 decreased in the second assay (i.e., from 5 to 2). This emphasizes the fact that both tests are easy

to interpret even for less experienced operators and that misinterpretations rapidly decrease over time. Nonetheless, the variability of the intensities reported by the different operators also highlights the increased subjectivity of both methods (Table 1).

For both tests, all false-positive results were classified as weak positives ("+"). A false-positive strain was consistently found by all operators with the Blue-Carba assays for an ACT-1-producing *E. coli*. Previous kinetic studies have shown that the plasmid-mediated AmpC (pAmpC) ACT-1 hydrolyzes slowly imipenem (Mammeri et al., 2010). It is to note that kinetic experiments have a much shorter time span compared to both tests. Additionally, we hypothesize that false-positive results can arise when different inoculum amounts are used in the test and the negative control solutions. This could explain the misclassification of the pAmpC MIR-1-producing *K. pneumoniae* as a positive result in the first assay but not in the second (Table 1).

As previously reported, class A and B carbapenemases yielded stronger results compared to class D enzymes regardless of the MICs attained for carbapenems (Table 1) (Österblad et al., 2014, Pasteran, Veliz, Ceriana, Lucero, Rapoport, Albornoz, Gomez and Corso, 2015, Pires, Novais and Peixe, 2013). Nevertheless, OXA-48 producers usually yielded stronger results with Blue-Carba than Carba-NP (e.g., 6 *vs*. 3 with "+++" for OP1 during the second assay, respectively). This difficult detection of OXA-48-like enzymes with Carba-NP is potentially linked to the B-PERII buffer. β-lactamases with lower imipenem hydrolytic efficiency produce less metabolites to overcome the buffer effect yielding weaker results. This has been reported as "buffer inhibition" which also justifies a different extraction solution used in the CarbaAcineto-NP that is mainly designed to detect OXA-type carbapenemases in *Acinetobacter* spp. (Dortet et al., 2014, Österblad, Hakanen and Jalava, 2014).

Interestingly, when comparing the agreement between the two tests considering only positive *vs.* negative results, the tests exhibit an almost perfect agreement [Kappa=0.91 (CI 95% 0.87-0.95)], emphasizing that both can be used to detect CPE given their high sensitivities and specificities. Additionally, this also highlights that the decreased cost of Blue-Carba can be extremely important in low income settings (Yusuf, Van Der Meeren, Schallier and Piérard, 2014).

Several potential sources of error have been identified. In our experience: *i*) the lack of standardization of the inoculum; *ii*) improper homogenization of the inoculum in the test solutions (Österblad, Hakanen and Jalava, 2014); and *iii*) improper storage of the test reagents (especially imipenem) can be linked to underperformance of both tests. Moreover, to improve detection, it is also suggested to increase the inoculum in either tests and also to perform them from specific media types and/or brands (Österblad, Hakanen and Jalava, 2014, Pires, Novais and Peixe, 2013, Tijet, Boyd, Patel, Mulvey and Melano, 2013). Despite the strong critics by some authors (Tijet, Boyd, Patel, Mulvey and Melano, 2013), it is undeniable that both methods can prove as an important clinical and epidemiological tool to be implemented in microbiology diagnostic labs. Additionally, the development of Carba-NP has encouraged the scientific community to improve and develop further quick alternative methods (Bakour et al., 2015, Bogaerts et al., 2015, Pasteran et al., 2015).

In conclusion, we demonstrated that both in-house Carba-NP and Blue-Carba tests are high sensitive and specific and thus suitable for rapid detection of CPE with an almost perfect agreement between the two tests. The simplicity of both tests makes them suitable for unexperienced operators readily identify carbapenemase production. Increasing the awareness of the possible errors on the test preparation and the

improvement of the protocol by standardizing the inoculum could be very important for increased sensitivity and specificity values.

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Table 1. Results obtained for the Carba NP and Blue-Carba tests performed using a collection of well-characterized strains (30 CPE and 33 non-CPE)

Acquired β-lactamases	Species (No. of strains with the same assay results)	Carba NP test						Blue-Carba test						MIC (µg/ml)			Reference or ATCC strain
		Assay 1			Assay 2			Assay 1			Assay 2						
		OP1	OP2	OP3	OP1	OP2	OP3	OP1	OP2	OP3	OP1	OP2	OP3	IMP	ERT	MEM	
Carbapenemase producers [a]																	
Class A (n=5)																	
KPC-2	*K. pneumoniae* (n=3)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	≥8	≥64	≥64	This study
	K. pneumoniae (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	1	16	4	This Study
	K. pneumoniae (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	1	8	2	ATCC BAA-1705
Class B (n=11)																	
IMP-1	*K. pneumoniae* (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	16	64	32	This Study
NDM-1	*K. pneumoniae* (n=5)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	≥1	≥4	≥2	This Study, (Principe et al., 2015)
	K. pneumoniae (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	≥64	≥64	≥64	This Study
	E. coli (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	16	≥64	≥64	This study
	E. coli (n=1)	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	8	≥64	≥64	ATCC BAA-2452
	E. cloacae (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	16	64	64	This Study
VIM-1	*K. pneumoniae* (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	8	0.5	1	This Study
VIM-2	*K. pneumoniae* (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	64	≥64	≥64	This Study
Class D (n=10)																	
OXA-48	*K. pneumoniae* (n=1)	+	+	+	+	+	+	+++	+++	+++	+++	+++	+++	4	32	16	This Study
	K. pneumoniae (n=1)	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	++	4	64	4	This Study
	K. pneumoniae (n=1)	+	++	+	++	+	++	++	++	+++	++	+++	++	4	≥8	≥16	This study
	K. pneumoniae (n=1)	+++	+++	++	+++	+++	++	+++	+++	+++	+++	++	+++	4	≥8	2	This Study
	K. pneumoniae (n=1)	++	++	+	++	++	+	+++	+++	+++	++	++	++	0.5	0.5	≤0.5	This Study
	K. pneumoniae (n=1)	++	++	++	+	++	+	++	+++	++	+++	+++	++	8	>= 8	2	(Giani et al., 2014)
	K. pneumoniae (n=1)	++	++	++	++	+++	++	++	++	++	++	++	++	4	>= 8	2	(Giani et al., 2014)
	E.coli (n=1)	+++	+++	++	+++	+++	++	+++	+++	+++	++	++	++	0.5	4	4	This Study
	E.coli (n=1)	+	+	+	++	++	++	++	++	++	+++	+++	+++	1	4	1	This Study
	Salmonella Kentucky (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	≤0.25	1	≤0.5	(Seiffert et al., 2014)
Class B + class D (n=3)																	
NDM-1 + OXA-48	*C. freundii* (n=1)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	4	≥8	2	This Study
	K. pneumoniae (n=2)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	≥4	≥4	≥16	This Study
Non-carbapenemase																	
Class A (n=9)																	
CTX-M-1	*E. coli* (n=2)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	(Endimiani et al., 2012)
CTX-M-1-like	*E. coli* (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	This Study
CTX-M-15-like	*K. oxytoca* (n=1)	-	-	-	-	-	-	-	-	+	-	-	-	≤0.25	≤0.25	≤0.5	This Study
SHV-12	*E. coli* (n=2)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	(Endimiani et al., 2012)
TEM-52	*E. coli* (n=2)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	
VEB-6	*P. mirabilis* (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	(Seiffert et al., 2013)
Class C (n=10)																	
ACT-1	*E. coli* (n=1)	-	-	-	-	-	-	+	+	+	+	+	+	≤0.25	≤0.25	≤0.5	b
CMY-2	*E. coli* (n=4)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	(Endimiani et al., 2012)
	P. mirabilis (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	2	≤0.25	≤0.5	This Study
DHA-1	*E. coli* (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	This Study
	K. pneumoniae (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	0.5	≤0.5	(Pilo et al., 2015)
FOX-1	*E. coli* (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	b
MIR-1	*K. pneumoniae* (n=1)	-	-	-	-	-	-	+	+	+	-	-	-	≤0.25	≤0.25	≤0.5	b
Class A + class C (n=1)																	
CTX-M-15-like + CMY-2	*E. coli* (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	This Study
No Acquired β-lactamases																	
	C. koseri (n=1)	-	-	-	-	-	-	-	-	-	-	-	+	≤0.25	≤0.25	≤0.5	This Study
	C. koseri (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	0.5	≤0.5	This Study
	E. coli (n=2)	-	-	-	-	-	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	ATCC 25922, (Endimiani et al., 2012)
	E. coli (n=1)	-	-	-	-	+	-	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	This Study
	E. aerogenes (n=1)	-	-	-	-	-	-	+	+	+	-	-	-	1	0.75	≤0.5	This Study
	E. cloacae (n=1)	-	-	-	-	-	-	-	-	+	-	-	-	≤0.25	2	≤0.5	This Study
	K. pneumoniae (n=5)	-	-	-	-	-	-	-	-	-	-	-	-	≤1	≤8	≤0.5	ATCC BAA-1706, This Study
	K. pneumoniae (n=1)	-	-	-	-	-	+	-	-	-	-	-	-	≤0.25	≤0.25	≤0.5	This Study

Note. "+++", strong positive; "++", positive; "+", weak positive; "-", negative; IMP, imipenem; ERT, ertapenem; MEM, meropenem

[a] Only carbapenemase genes are shown; [b] A kind gift from Robert A. Bonomo, Cleveland, OH, USA.